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Permanent impairment in the feeding behavior of grayling (*Thymallus thymallus*) exposed to methylmercury during embryogenesis

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Abstract

Embryos of grayling (*Thymallus thymallus*) were exposed to different concentrations of methylmercury (0.16, 0.8, 4.0 and 20 μg Hg l^{-1}) during the first 10 days of development. The exposure resulted in body concentrations in the newly hatched fry of 0.09, 0.27, 0.63 and 3.80 μg Hg g^{-1} wet wt., respectively. A control group had a body concentration of 0.01 μg Hg g^{-1}. Morphological disturbances were only found in the highest exposure group. Three years later, at a size of 13.8 ± 0.8 cm, the different groups were tested for sublethal toxicant effects on foraging behavior. In the first series of experiments we tested the foraging efficiency of the fish when kept alone for 5 min in small flow-through aquariums. In the second series of experiments we tested the competitive ability of eight individuals from an exposed group vs. eight individuals from a control group when kept together for 30 min in a 300-l aquarium. In both experiments live *Dapnia magna* were used as prey. We found impaired feeding efficiencies and reduced competitive abilities in grayling from the exposed groups which as yolk-fry had Hg concentrations of 0.27 μg g^{-1} or more. In the foraging efficiency experiments these groups were 15–24% less efficient as compared to the control group. In the competitive ability experiments the control group caught two to six times as many preys as these exposed groups. Such harmful body concentrations of Hg (> 0.27 μg g^{-1}) may be found in eggs from piscivorous fishes in lakes receiving diffuse atmospheric depositions of mercury. We suggest such concentrations may have ecological consequences by reducing the fitness of the affected populations. © 1998 Elsevier Science B.V.

Keywords: *Thymallus thymallus*; Methylmercury; *Dapnia magna*

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1. Introduction

Mercury contamination of freshwater fish is a severe problem in both Fennoscandia and North America. The scientific focus on mercury in aquatic ecosystems has largely been motivated by the health risks of consuming contaminated fish, because large numbers of remote lakes contain fish with mercury concentrations often exceeding those considered safe for human consumption (0.5–1.0 $\mu g\ g^{-1}$) (Andersson et al., 1987; Sorensen et al., 1990; Verta, 1990; Wiener and Stokes, 1990). Mercury in fish exists primarily as methylmercury (MeHg). This is an extremely dangerous neurotoxin, especially for embryos, as it is known to interfere with the development of the central nervous system by causing abnormal neuronal migration and damage to the microtubulus in neurons and astrocytes (Choi, 1983). Typically lethal mercury concentrations in adult fish have been reported to lie between 10 and 30 mg kg^{-1} (Wiener and Spry, 1996). However, little is known about the sublethal effects of MeHg on fish behavior, as the primary scientific efforts have been addressed to the study of standard physiological endpoints, such as reduced survival, growth and reproduction. We believe the study of sublethal effects on fish foraging behavior may be a more sensitive test and also have more ecological relevance than standard toxicological tests (Wiener and Spry, 1996). We therefore incubated eggs from an uncontaminated population of grayling (*Thymallus thymallus*) in different concentrations of MeHg for 10 days, reared them in an uncontaminated environment for 3 years and then tested their feeding efficiencies and competitive abilities compared to a control group.

2. Materials and methods

2.1. The fish

The grayling used in these experiments originate from the sub-alpine lake Aursjøen, southern Norway. In July 1991, we caught by electrofishing 12 pairs of sexually mature, ripe male and females on the spawning grounds in the Kvita tributary. The stripped eggs from each female were dry-fertilized with milt from two to three males to ensure successful fertilization. The eggs were then put in perforated plastic boxes and allowed to swell in the river water (12°C) for 2 h. After hardening, the eggs were transported in ice-cooled freight boxes (4°C) to the University of Oslo for further treatment. In the laboratory, we randomly assigned eggs to five different test groups of approximately 1800 eggs each. Each test group was evenly distributed in a single layer of eggs on the bottom of six glass cups (diameter 10 cm, height 1.5 cm) and put into a flow-through aquarium for exposure to the toxicant. The whole process from the fertilization of the egg to their final placement into the aquaria took approximately 12 h.

We had in advance prepared one control and four exposure aquaria. The aquaria were made of glass and contained 40 l of water. A five-channel peristaltic pump supplied unpolluted water to each aquarium with a flow of 0.30 l h^{-1}. The water source was Lake Maridalsvannet, which is used as a drinking water reservoir and is a typically soft water lake (pH = 6.3, Ca = 2.7 mg l^{-1}, TOC = 3.4 mg l^{-1}, Hg < 1 ng l^{-1}). The toxicant was supplied by peristaltic pumps with a flow of 4 ml h^{-1} to the four exposure aquariums. All hoses were made of silicon rubber. Air-bubbling ensured the water to be well mixed and aerated. The stock solutions of the toxicant were prepared from CH_3HgCl dissolved in distilled water and the four nominal exposure concentrations increased by a factor of 5, from 0.16 to 20 μg Hg l^{-1}. The eggs were incubated at 14–15°C. During the incubation period, some of the eggs were infected by fungi (*Saprolegnia* sp.) and at day 6 after fertilization we had to treat all groups with malachite green (1 ppm $\mu g\ l^{-1}$ for 15 min) to avoid unacceptable losses. We inspected the aquariums each day, removed dead or infected eggs and adjusted the water flow if necessary. In the following we name the different test groups and exposure aquariums from A (control) to E (highest exposure).

At day 13, after flushing the aquariums with unpolluted water, we removed approximately 100 newly hatched embryos from each group for analysis of mercury. The pooled samples ($\approx$ 1 g each) were acid-digested before being analyzed on a

cold vapor atomic absorption spectrophotometer with a hydride generator (detection limit: 0.01 $\mu g\ g^{-1}$).

After hatching, all free-living normal looking embryos were transferred to larger tanks for exogenous feeding. We used live zooplankton from a nearby pond and nauplii larvas of *Artemia saliens* as prey during the first phase of exogenous feeding. After termination of the start-feeding period we reared the fish in relatively cold water (2–10°C) and fed them with small rations of commercial salmon food (EWOS®). Occasionally (approximately once per week) they were also given live *Daphnia magna*, to keep them familiar with this prey. The cold water regime and small food rations resulted in a low growth rate and kept the fish at an appropriate size for the later experiments. After 3 years, when the fish had reached a length of 10–16 cm, we started the feeding experiments. The temperature regime and growth rate experienced during the breeding period is not uncommon in Arctic or Alpine grayling populations.

2.2. The foraging experiments

We planned two different series of experiments to reveal possible effects of MeHg on the fish feeding behavior. In the first series we wanted to test the foraging efficiency of the fish when kept alone in small flow-through aquariums. In the second series we wanted to test the competitive ability of an exposed group vs. a control group when several individuals were kept together in a large aquarium. As prey in both experiments, we used live *Daphnia magna*. To obtain prey of similar size, we carefully sifted them, using the fraction retained on a 0.85-mm mesh but passing through a 1.00-mm mesh. Measurements of 50 prey showed that the mean length from the head to the abdominal spine was 1.38 ± 0.26 mm (mean $\pm$ S.D.). Cladocerans of this size group is an important food item for the parent grayling population in lake Aursjøen (Haugen and Rygg, 1996). The fish used in the foraging experiments belonged to the modal length groups from the different test groups and their mean sizes were 13.8 ± 0.8 cm.

In the foraging efficiency experiments we used fish marked with an individual color code, to make it possible for us to identify them during the experiments. The pigments (red and blue drawing ink) were carefully injected at the basis of their fins, using a jet injector. After marking, the fish were allowed to acclimatize in the experimental aquariums for approximately 1 week and they were fed daily with artificial food and daphnias. One day before the start of an experiment, we transferred the fish to an empty aquarium, put under an illuminated white tent. The illuminance at the water surface was 3.5 μmol photon s^{-1} m^{-2}. The aquariums measured $50 \times 20 \times 20$ cm, they had one inlet and two outlet hoses connected to the end walls, maintaining a water level of 11 cm. A pump supplied them continuously with 5.5 l of water per min. The temperature in the aquariums was 6.0 ± 0.6°C during the acclimatization period and the experiments. The estimated half-time of prey density in the aquariums (without fish) were 122 ± 3 s (non-linear regression of experimental data, assuming an exponential model). Each experiment lasted for 5 min and for the first and every 30 s we added 10 daphnias to a funnel connected to the inlet hose. The outlet water was sifted through a fine mesh, retaining escaped prey. During the experiments we observed the fish through a hole in the tent. We stopped the experiment if the fish did not start to feed after 30 s, or if it suddenly ceased to eat or seemed frightened. At the end of an experiment we took the fish out of the aquarium, flushed the aquarium and counted the number of escaped prey. The order of the single experiments were not completely randomized, as we first tested the control group (A) and the highest exposure group (E) and then later tested individuals from the other exposed groups along with individuals from the control group. There was no systematic drift in the feeding efficiency of the control group throughout the experimental period, thus we regard the experimental conditions to be stable. We compared the group means of number of preys eaten by one-way analysis of variance and post-hoc tests. We believe that the turbulent water and short resident time of prey in these aquaria make them a more challenging and realistic environ-

ment for drift-feeders than more static test systems.

In the competitive ability experiments we let fish from an exposed group forage together with fish from the control group in a large aquarium with a constant input of daphnias. Our hypothesis was that this could be a more sensitive test of differences in foraging efficiency, because of interference during competition for food (Milinski, 1988). The experimental aquarium measured 116 $\times 50 \times 58$ cm and was filled with 300 l of water. The illuminance at the water level was 4.5 μmol photon s^{-1} m^{-2}. The temperature during the experiments was 8.0°C. Before an experiment, we transferred eight fish from an exposed group and eight fish from the control group to this aquarium. To make it possible to distinguish between individuals from the different groups, they were color marked by injecting a red or blue pigment spot at the basis of the caudal fins. The fish were then allowed to acclimate in the experimental aquarium for 3–5 days and they were not fed the last 24 h before an experiment.

During the competitive ability experiments, water circulated through the aquarium and a system of pumps and reservoir tanks for prey. The water flow was 5 l min^{-1} and the aquarium received a stable input of 50 daphnias per minute. Each experiment lasted for 20 min and the fish were then immediately killed by a blow to the head. We dissected their stomachs and esophagus and counted the number of prey eaten. We did not include group B in this experiment, as there was no difference between this group and the control in the feeding efficiency experiment. To achieve an acceptable power of the statistical analyses, we had to make replicate experiments when testing the exposure groups C and D vs. the control. The results from the replicate experiments were analyzed by two-way analysis of variance (mixed model), allowing for differences and interactions between treatments (fixed effects) and replicates (random effects) (Sokal and Rohlf, 1981). A one-way analysis of variance was performed for the experiment without replicates. The statistical analyses were done by the computer program JMP v. 3.1 (SAS Institute Inc., Cary, NC).

3. Results

3.1. The breeding period

The first hatchings occurred 10 days after fertilization in groups D and E. The other groups started to hatch 1 day later. Fifty percent hatching occurred 2 days after the onset of hatching. At day 13, more than 90% of the live eggs in all groups had hatched, except for the high exposure group E. At this point we stopped the MeHg exposure. Numerous embryos from group E seemed unable to hatch successfully and several of the free-living embryos were malformed. The apparent malformations were scoliosis (lateral curvature of the spine and unable to uncurl after hatching) and jaw deformities. At day 15 approximately 80% of the live eggs in group E had hatched. As it was evident that no more viable embryos would hatch, we terminated this part of the experiment. Clearly, the mortality of group E during the hatching period was larger than in the lower exposure groups (60% vs. 30–15%). However, a closer examination of the mortality rates is not very relevant, because of the losses due to the Saprolegenia infection.

The analyses of the 13-day-old newly hatched embryos showed that the body concentrations of Hg were 0.01, 0.09, 0.27, 0.63 and 3.8 μg g^{-1} (wet wt.) for groups A–E, respectively.

During the 3-year long breeding period previous to the foraging experiments, we found no noticeable growth differences between the different groups, except for a few small individuals from the high exposure E who had developed jaw deformities. The fish used in the foraging experiments belonged to the modal length groups from each batch. Their mean length were 13.8 ± 0.8 cm and we found no significant length differences between the groups.

3.2. Feeding efficiency

In the feeding efficiency experiments, generally the fish started to forage immediately after the first batch of prey was added. Usually, the fish positioned themselves in the middle of the aquar-

ium, with the head pointing in the direction of the water inlet and made rapid bursts towards preys drifting by. In these experiments we found an adverse effect of the MeHg treatment on fish behavior. The number of prey caught seemed to decrease with increasing MeHg exposure (Fig. 1) and an analysis of variance showed significant differences between treatments in the numbers of prey consumed ($F = 9.62$, d.f. = 4,47, $P < 0.001$). A Dunnett's test showed the means of group C, D and E to be significant smaller than the mean of the control group A ($P < 0.05$) and these groups were on average 15–24% less efficient in their foraging activity than the control group. A closer inspection of the data revealed that the variation around the means seemed to increase with the mercury dose. A test for equal variances showed this effect to in the borderline of significance (O'Brian test: $F = 2.52$, d.f. = 4,47, $P = 0.06$). We therefore do not feel confident that this assumption for an analysis of variance is not violated. However, a Welch Anova allowing for inhomogeneous variances, showed the sample means to be significantly different ($F = 8.79$, d.f. = 4,18.46, $P < 0.001$).

During the feeding experiments we had to stop some of the trials, or reject the results, because the fish behaved aberrant (see methods). The numbers of dismissed trials in the different groups were: 1 (A); 0 (B); 3 (C); 3 (D); and 4 (E). These figures indicate that the frequency of dismissed trials increased in the high exposed groups and a likelihood ratio test for independence across each level indicated a borderline significance ($\chi^2 = 8.72$, d.f. = 4, $P = 0.07$). However, the numbers of dismissed trials are sparse and the significance test has to be viewed with caution.

3.3. Competitive ability

In the competitive ability experiments the first fishes started to feed within 30 s after the first prey was introduced to the aquarium. After 2 min most fishes had adopted a saltatory search behavior, which is a typical feeding behaviour for visually feeding planctivorous fishes. This behavior is characterized by moving in a relatively rapid stop-and-go fashion, while repositioning by turning a small angle after each stop (O'Brian et al., 1990). Again we found an adverse effect of the MeHg treatment on fish behavior and all experiments showed fish from the exposed groups to be inferior competitors as compared to fish from the control (Fig. 2).

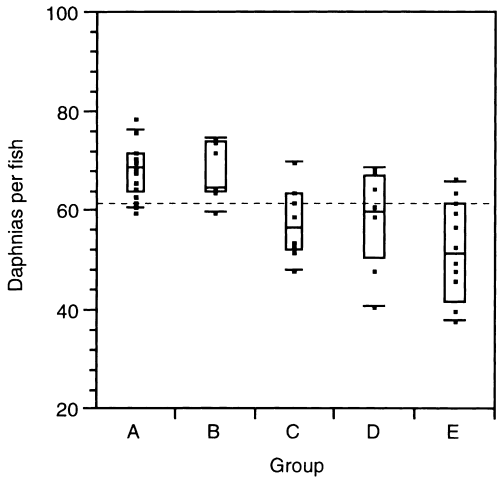

Fig. 1. Box plots of the number of preys caught in the feeding efficiency experiments. Boxes mark median and interquartiles ranges, while horizontal lines above and below the boxes mark the 10 and 90% percentiles. The dashed line marks the grand mean for the whole sample. The arithmetic means, standard deviation and number of trials were as follows: (A) 68.2 ± 5.2, $n = 17$; (B) 67.9 ± 5.6, $n = 7$; (C) 57.8 ± 7.3, $n = 8$; (D) 58.8 ± 9.8, $n = 8$; (E) 51.9 ± 10.2, $n = 12$.

In the first experiment we tested the highest exposure group E vs. the control group A and the results showed that the control group on average caught more than six times more prey than the exposed group: 65.9 ± 12.3 vs. 10.5 ± 6.7 (mean $\pm$ S.E.). The difference was highly significant ($F = 15.38$, d.f. = 1,14, $P = 0.002$).

In the second experiment we tested the exposed group D vs. the control and we made two replicates to increase the power of the test. An analysis of variance showed: (1) no significant interactions between treatments and replicates; (2) no significant difference between replicates; (3) significant differences between groups ($F = 6.49$, d.f. = 1,30, $P = 0.016$). The group means of the pooled data showed that the control group

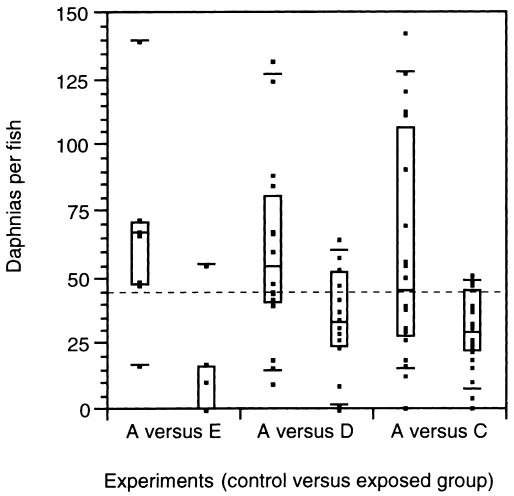

Fig. 2. Box plots of the number of prey caught in the competitive ability experiments. Boxes mark median and interquartiles ranges, while horizontal lines above and below the boxes mark the 10 and 90% percentiles. The dashed line marks the grand mean for the whole sample. (See text for arithmetic means and standard errors.)

had caught approximately twice as many prey as the exposed group: 62.8 ± 10.7 and 25.8 ± 3.5 for groups A and D, respectively.

In the third experiment we tested the low-exposed group C vs. the control and we made three replicates. An analysis of variance showed: (1) no significant interactions between treatments and replicates; (2) no significant difference between replicates; (3) significant differences between groups ($F = 10.41$, d.f. = 1,46, $P = 0.002$). Again, the group means of the pooled data showed that the control group had caught almost twice as many prey as the exposed group: 60.3 ± 6.5 and 30.5 ± 6.5 for groups A and C, respectively.

4. Discussion

In a recent review of the toxicological significance of Hg for freshwater fish, Wiener and Spry (1996) point out the need for more specific knowledge about the sublethal effects of MeHg on fish behavior. They suggest that the neurotoxic effects of MeHg may impede the wild fish to locate, capture, handle and ingest prey, causing starvation in mercury-contaminated lakes. In their review the conclude that 'Sublethal and lethal effects on fish embryos are associated with mercury residues in eggs that are much lower than (perhaps 1–10%) the residues associated with toxicity in adult fish'. We believe our present study strengthens their hypothesis. Our results show that fish developed from embryos with MeHg concentrations equal or larger than 0.27 mg Hg kg^{-1} may evolve permanent impairments in their foraging behavior. During competition in still water, these exposed fishes caught 50% or less number of prey than the control group. When they fed alone in a small flow through aquarium they were 15–24% less efficient in their foraging activity than the control group.

However, a main point in our argument and critically for the relevance of our experiments, is whether the experimentally-induced MeHg concentrations in our study reflect actual levels found in natural populations. In nearly pristine or lightly contaminated lakes and streams, concentrations of total mercury may are often in the range of 0.6–4 ng l^{-1}, while the MeHg concentrations in naturally oxic fresh waters without direct sources typically range from 0.01 to 0.8 ng l^{-1} (Wiener and Spry, 1996). This is far below the MeHg concentrations used in our experiments (0.16–20 μg Hg l^{-1}). However, our intention was not to create a realistic waterchemical environment, but to efficiently make the developing eggs accumulate a wide range of sublethal doses of MeHg.

Nearly all (95–99%) of the mercury in fish is MeHg (Hucabee et al., 1979; Grieb et al., 1990; Bloom, 1992). The gonads of mercury-exposed female fishes contain smaller concentrations of mercury than most other organs and tissues. Unfortunately, the data are few, but available data from McKim et al. (1976) indicates that the mercury concentration in embryos of MeHg-exposed brook trout (*Salvelinus fontinalis*) is approximately 20% of that in the maternal axial muscle tissue. Thus, according to our results, the offspring of female fish with Hg concentrations in muscle exceeding 1.35 μg g^{-1} are at risk of having reduced feeding abilities and may be inferior competitors as compared to unexposed individuals. This is alarming as such concentrations

are not uncommon in piscivorous fish from susceptible localities (Wiener and Spry, 1996).

Previous studies by McKim et al. (1976) on the toxicity of MeHg to fish have shown that the no-effect level or maximum acceptable concentration for chronic exposure is between 0.93 and 0.29 μg Hg l^{-1} and they conclude that residues in fish between 3 and 5 mg Hg kg^{-1} would be without direct toxic effect on the fish. This is well above the toxic levels we found for developing embryos (0.27 μg g^{-1}), or the estimated corresponding maternal muscle concentration (1.35 μg g^{-1}, using a concentration ratio of 1:5). However, the effects measured by McKim et al. (1976) were related to growth, reproduction and survival, which is not as sensitive as behavioral parameters (Atchinson et al., 1987).

In an experiment by Weis and Weis (1994), the authors exposed embryos of mummichog (*Fundulus heteroclitus*) to 2–10 μg l^{-1} MeHg during development (14 days). These are concentrations well beyond those which cause teratological effect in this species. The authors found a transitory effect on the prey-capture ability of the larvas, but after approximately 1 week the exposed individuals had the same prey-capture abilities as the control. The authors conclude that the exposure may have caused a developmental retardation in the neurological development, which subsequently was compensated for. The concentrations used are in the same range that resulted in a significant and permanent reduction in the prey-capture abilities in our experiments. Thus it seems as if differences between species or environments (marine vs. freshwater) may cause very different responses to MeHg exposure. Our results are more in accordance to those of Birge et al. (1979), who found lethal effects of inorganic mercury exposure to coincide with mercury concentrations of approximately 0.1 μg g^{-1} in embryos of rainbow trout (*Oncorhynchus mykiss*).

The lack of pronounced growth differences after the 3 years breeding period may seem inconsistent with the results from the foraging experiments. The similar growth pattern indicate that the effects of methyl mercury did not restrain the exposed groups in their foraging activity when they fed on artificial food pellets in the breeding tanks. However, catching a slowly sinking food pellet or picking it up from the bottom of a breeding tank, is a far less challenging exercise than capturing a swimming daphnia in a strong turbulent current or under intense competition such as in our foraging experiments. We suggest that the neurological damages due to the methyl mercury exposure first make their appearance under situations demanding capable coordination and perception skills.

We did not measure the mercury content of fish after the behavioral experiments, but we have no reason to believe that there should be any significant differences between the groups. The weight increased from approximately 0.01–17 g in the test fish during the breeding period and the initial concentration differences should therefore have levelled out. Most likely were the mercury concentrations in the test fish close to those found in other similar sized salmonids from uncontaminated environments (0.01–0.05 μg g^{-1}; Rognerud et al., 1996).

It may also be questioned whether the antifungal treatment of eggs with malachite green may have interfered with our experiments, as malachite green is suspected to be a teratogenic substance. We cannot rule out such an effect, but regard it as unlikely to have contributed to our results, as all groups including the control were given identical treatments.

To conclude, our results show that grayling exposed to MeHg during embryogenesis, resulting in a Hg concentration of 0.27 μg Hg g^{-1} or more in the yolk-fry, had a permanent impairment of their feeding efficiency and competitive ability. Fish in susceptible localities which receive diffuse atmospheric depositions of Hg may accumulate such concentrations of MeHg in their gonads, resulting in a reduced fitness of the affected populations.

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